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## ***In vitro* screening of the biological activity of combined extracts of two medicinal plants and their standardization by validated analytical methods using standard markers**

**Jayaprakasam Rajendran and Ravi Thengungal Kochupappy**

### **Abstract**

The aim of the present study is to carry out *in vitro* antiarthritic and antigout activity of the combined extracts of two plants namely *Mangifera indica* and *Hygrophila spinosa* and their standardization by HPTLC and HPLC methods using appropriate markers. The synergistic properties of the extracts pertaining to antiarthritic and Antigout activities were evaluated. The mobile phase for HPTLC was optimized as toluene: ethyl acetate: methanol, 15:3:1.5% v/v/v. The presence of mangiferin was detected at 270nm under UV light and the lupeol was detected after derivatization with Liebermann burchard reagent at 366nm. In the HPLC method, the optimized solvent system used was acetonitrile: methanol: 20mM potassium di hydrogen phosphate 30:20:45% v/v/v and detection was made at 210nm. The combined extracts produced better activity than the individual extracts of the respective plants. Hence, these combined plant extracts were standardized, quantified and validated by newly developed HPTLC and HPLC methods as per ICH guidelines.

**Keywords:** *Mangifera indica*, *Hygrophila spinosa*, antiarthritic, antigout, HPTLC, HPLC

### **Introduction**

Traditional systems of medicine have been steadily gaining importance and acceptance all over the world. Consequently, plant materials and herbal based drugs derived from them represent a substantial proportion of the current global drug market. In this scenario, there is a need to ensure that herbal drugs and preparations containing them possess optimum and consistent quality. Hence, there is a need to create and maintain a comprehensive quality assurance system. Quality control and standardization of herbal drugs are considered to be one of the major issues in herbal drug development [1].

Chemical analyses of plants show the presence of therapeutically important constituents usually in combination with many inert substances. The active principles are extracted from the plants and purified for therapeutic utility based on their selective pharmacological activity. Medicinal plants play an important role in the development of potent therapeutic agents. The use of herbal medicines is becoming popular due to the toxicities and side effects associated with allopathic medicines [2].

Rheumatoid arthritis, one of the common auto immune diseases, is a chronic, progressive and systemic inflammatory disorder affecting synovial joints producing symmetrical arthritis leading to joint degeneration [3]. Gout is a common metabolic disorder in humans which is associated with elevated uric acid level in the blood leading to the deposition of urate crystals in the joints and kidney. For assessment of the extent of antiarthritic activity, inhibition of protein denaturation, Proteinase inhibition and Hyaluronidase inhibitory assay and for antigout activity, xanthine oxidase inhibitory assay were carried out for the combined extracts [4].

Standardization of plant extracts with the help of markers is an essential procedure for ensuring the quality control of the herbal drugs which would lead to increased global acceptance of them. Standardisation is defined as "formulation of standards for a substance or for a procedure". Standardization is an essential process for ensuring the quality control of the herbal drugs. In the development of botanical drugs, standardisation refers to a set of technique or standards that are applied to the manufacture of herbal formulations. According to the American Herbal Products Association (AHPA), "Standardisation refers to the body of information and controls necessary to produce materials of reasonable consistency. This is achieved through minimizing the inherent variation of natural product composition through quality assurance practices applied to agricultural and manufacturing processes" [5].

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Nowadays, there are increasing trends to follow herbal lifestyle to adopt herbal dietary choices for human welfare and choosing herbal drugs for their primary healthcare needs. The toxic side effects of allopathic medicines and lack of these medicines for many chronic ailments have led to the re-emergence of herbal medicines as drugs of choice. Consequently, the assurance of safety, quality and efficacy of herbal products has become an important issue and development of standards for plant-based drugs is a challenging task that needs innovative and creative approaches, different from the routine methods.

The bark of *Mangifera indica* and aerial parts of *Hygrophila spinosa* were selected for screening them for antiarthritic and antigout activities. *Mangifera indica* (Family: Anacardiaceae) contains mangiferin, alanine, glycine,  $\alpha$ -aminobutyric acid, kinic acid and shikmic acid. Bark is used as astringent; it is used in the treatment of diphtheria and rheumatism; it has tonic action on the mucous membrane. Mangiferin has shown antibacterial and antioxidant activities. It also exhibited in vitro inhibitory effects on II 5 $\alpha$ -reductase and gastroprotectivity and antidiabetic effects on rodents. [6-15] Chemically it is 1,3,6,7-tetrahydroxy-2-[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl] xanthene-9-one; 1,3,6,7-tetrahydroxy-xanthene-9-one. It has molecular Formula C<sub>19</sub>H<sub>18</sub>O<sub>11</sub> and Molecular Weight 422.33 (Figure 1)

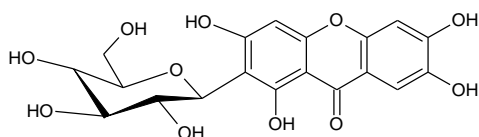


Fig 1: Structure of Mangiferin

Aerial parts of *Hygrophila spinosa* which belongs to the family, Acanthaceae was found to contain phytosterols, polyphenols, proanthocyanins, alkaloids, flavonoids, terpenoids, glycosides, saponins, histidine, lysine and lupeol. Lupeol has antiarthritic, antiprotozoal, antimicrobial, antihelminthic, anti-inflammatory, anti-hyperglycaemic, antitumor and chemo preventive activities. As an anti-inflammatory agent, lupeol acts primarily on the interleukin system. Lupeol is found to decrease IL-4 (interleukin 4) production by T-helper type 2 cells. [16-23] Chemically it is (3 $\beta$ ,13 $\xi$ )-Lup-20(29)-en-3-ol with the molecular formula C<sub>30</sub>H<sub>50</sub>O and molecular weight of 426.72 (Figure 2).

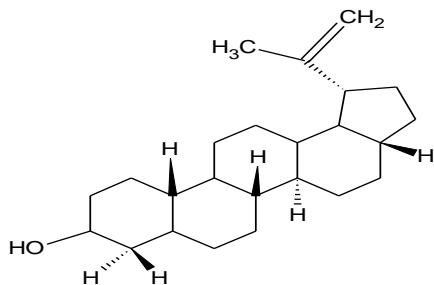


Fig 2: Structure of Lupeol

In the present study, methanolic extracts of the individual plants exhibited excellent antiarthritic and antigout activities individually and also in combination. They were found to possess significant antiarthritic and antigout activities as described in the previous section. Hence, it is essential to establish the standards of the mixture of the combined extracts using respective markers. The HPTLC and HPLC

methods were developed and validated for assuring the presence of active principles by using markers and to quantify them in the combined extract mixtures.

## Materials and Methods

### Chemicals and reagents

Mangiferin and Lupeol were purchased from Sigma Aldrich, India. AR/HPLC grade methanol, toluene, acetonitrile, ethyl acetate, acetic anhydride, concentrated sulphuric acid and ethanol were supplied by S.D. Fine Chemicals Ltd., and Merck Pvt. Ltd., Mumbai.

### Plant material

The plant materials collected were confirmed as *Mangifera indica* and *Hygrophila spinosa* by Dr. C. Kunhikannan, Scientist E, Biodiversity division, Institute of Forest Genetics and Tree Breeding, (Indian Council of Forestry Research and Education) Coimbatore, Tamil Nadu, India.

### Instruments

HPTLC was performed in Camag HPTLC System equipped with Linomat 5 sample applicator, twin trough plate development chamber, TLC Scanner 3 with WinCATS software. RP-HPLC was performed in Shimadzu HPLC system with LC AT10 VP Pump, SPD M 10 AT VP Detector and CLASS M 10A software. The stationary Phase used was Phenomenex, Luna, C<sub>18</sub> column (150 x 4.6mm, 5 $\mu$ ).

### Extraction

The bark of *Mangifera indica* and aerial parts of *Hygrophila spinosa* were individually ground well for extraction process. 1000g of the powder of each drug was used and the extraction was carried out successively by continuous hot percolation method using soxhlet apparatus using solvents of increasing polarity such as petroleum ether, chloroform and methanol at a temperature of 30-45°C for three days. Volume of solvent used was 1000ml.

### Antiarthritic studies of plant extracts

#### Model 1: Inhibition of protein denaturation

##### Preparation of reagents

Bovine serum albumin (BSA, 5%) was prepared by dissolving 5g of BSA in 100ml of water. Phosphate buffer saline of pH 6.3 was prepared by dissolving 8g of sodium chloride, 0.2g of potassium chloride, 1.44g of disodium hydrogen phosphate and 0.24g of potassium dihydrogen phosphate in 800ml distilled water. The pH was adjusted to 6.3 using 1N HCl and the volume was made up to 1000ml with distilled water.

##### Preparation of extracts

Sufficient quantities of *Mangifera indica* and *Hygrophila spinosa* extract material were dissolved in methanol to get concentrations of 25, 50, 75 and 100 $\mu$ g/ml.

##### Procedure

The reaction mixtures were prepared by adding bovine serum albumin (5% aqueous solution) to 0.05ml of methanolic extracts of *Mangifera indica* and *Hygrophila spinosa* and standard diclofenac (25, 50, 75 and 100 $\mu$ g/ml) individually in test tubes. The total volume of each reaction mixture was fixed as 0.5ml. pH was adjusted to 6.3 using 1N HCl. The samples were incubated at 37°C for 20 minutes and then maintained at 57°C for 3 min. After cooling the samples, 2.5ml of phosphate buffer saline (pH 6.3) was added to each tube. Absorbance was measured spectrophotometrically at

371nm and 359nm respectively for *Mangifera indica*, and *Hygrophila spinosa*.

A control test was carried out using 0.05ml of distilled water instead of the extracts while product control tests lacked bovine serum albumin. The percentage inhibition of protein denaturation was calculated as follows

Percentage inhibition (%) =  $100 \times \frac{\text{absorbance of control} - (\text{absorbance of test} - \text{absorbance of product control})}{\text{absorbance of control}}$

The control represents 100% protein denaturation. The results of the individual extracts were compared with the results of diclofenac treated samples.

## Model 2: Proteinase inhibitory activity

### Preparation of reagents

About 25mM of tris-HCl buffer pH 7.4 was prepared by dissolving 3.94g in 800ml of de-ionised water, the pH was adjusted to 7.4 using 1M HCl and the volume was made up to 1000ml with deionized water. A 0.8 % (w/v) solution of casein was prepared by dissolving 0.8g of casein in 100ml of distilled water. A 0.06mg/ml of the trypsin solution was prepared by dissolving the required quantity of trypsin sample in buffer solution. The methanolic extracts were prepared to give 10, 20, 40, 80 and 100µg/ml concentrations.

### Procedure

The reaction mixtures were prepared by adding 0.06mg of trypsin and 1ml of 25mM tris-HCl buffer (pH 7.4) to 1ml of each solution of *Mangifera indica* and *Hygrophila spinosa* and standard diclofenac to make up a total volume of 2ml. The mixtures were incubated at 37°C for 5 minutes and then 1ml of 0.8% w/v casein was added to each mixture. The mixtures were incubated for additional 20 minutes. About 2 ml of 70% perchloric acid was added to each test tube to terminate the reaction. The cloudy suspension obtained was centrifuged.

Absorbance of the supernatant was read at 217 and 208nm for *Mangifera indica* and *Hygrophila spinosa* respectively against buffer as control. The percentage inhibition was calculated as follows:

Percentage inhibition (%) =  $100 \times \frac{\text{absorbance of control} - (\text{absorbance of test} - \text{absorbance of product control})}{\text{absorbance of control}}$

## Model 3: Hyaluronidase inhibitory activity

### Preparation of reagents

A 5% DMSO was prepared by mixing 5ml of the DMSO solution in 100ml of water. About 0.1M ammonium acetate buffer of pH 3.5 was prepared by dissolving 2.5g of ammonium acetate in 2.5 ml of water, 3.8ml of 7M HCl was added, the pH was adjusted to 3.5 with 2M HCl and finally the volume was made upto 1000ml with water. 4g of p-dimethyl amino benzaldehyde was dissolved in 350ml of 100% acetic acid and 50ml of 10N hydrochloric acid was added to get p-dimethyl amino benzaldehyde reagent.

### Procedure

Methanolic extracts prepared from *Mangifera indica* and *Hygrophila spinosa* were examined for their effects on the enzyme hyaluronidase. About 50µl of bovine hyaluronidase (7900 units/ml) was dissolved in 0.1M acetate buffer (pH 3.5) and mixed with 100µl of a designated concentration of sample (methanolic extract of the two plants) dissolved in 5% dimethyl sulfoxide. Then the mixture was incubated in a water bath at 37°C for 20 minutes. The control group was

treated similarly taking 100µl of 5% DMSO instead of the sample.

About 100µl of 12.5mM CaCl<sub>2</sub> solution was added to the reaction mixture and then the mixture was incubated in a water bath at 37°C for 20min. The Ca<sup>2+</sup> activated hyaluronidase was treated with 250µl of sodium hyaluronate (1.2mg/ml) dissolved in 0.1M acetate buffer (pH 3.5) and then the mixture was incubated in a water bath at 37°C for 40min. About 100µl of 0.4N NaOH and 100µl of 0.4N K<sub>3</sub>BO<sub>3</sub> were added to the reaction mixture and then they were incubated in a boiling water bath for 3 minutes. After cooling to room temperature, 3ml of p-dimethyl amino benzaldehyde solution was added to the reaction mixture which was then incubated in a water bath at 37°C for 20 min.

Absorbance of the reaction mixture was read at 404nm by using a spectrophotometer. The percentage inhibition was calculated as:

Percentage inhibition (%) =  $\frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100$

## Antigout activity of the extracts of selected plants

### Assay for xanthine oxidase inhibitory activity

#### Preparation of reagents

Phosphate buffer of pH 7.5 was prepared by dissolving 8g of sodium chloride (NaCl), 0.2g of potassium chloride (KCl), 1.44g of disodium hydrogen phosphate (Na<sub>2</sub>HPO<sub>4</sub>) and 0.24g of potassium dihydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>) in 800ml distilled water. The pH was adjusted to 7.5 using 1N HCl and the volume was made up to 1000ml with distilled water.

#### Method of preparation of the extracts

Sufficient quantity of *Mangifera indica* and *Hygrophila spinosa* methanolic extracts were dissolved in 5% DMSO to prepare solutions of concentrations 10, 20, 40, 80 and 100µg/ml.

### Procedure

The extracts of the selected plants *Mangifera indica* and *Hygrophila spinosa* were assayed for their *in vitro* xanthine oxidase inhibitory activity.

The xanthine oxidase inhibitory (XOI) activity was assayed spectrophotometrically under aseptic conditions using xanthine as substrate. The assay mixture consisted of 1ml solutions each of the 10, 20, 40, 80 and 100µg/ml concentrations of the specific extract, 2.9 ml of phosphate buffer (pH 7.5) and 0.1ml of xanthine oxidase enzyme solution (0.1units/ml in phosphate buffer pH 7.5) which was prepared immediately before use. After preincubation at 25°C for 15min, the reaction was initiated by the addition of 2ml of the substrate solution (150µM xanthine in the same buffer). The assay mixture was incubated at 25°C for 30 minutes. The reaction mixture was then assayed by the addition of 1ml of 1N HCl and measurement of the absorbance at 290nm using a spectrophotometer. Allopurinol, a known inhibitor of XO (10-100 µg/ml), was used as the positive control.

XOI activity was expressed as the percentage inhibition of XO and is calculated as:

Percentage inhibition (%) =  $\frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100$

## Standardization of combined extracts of the selected plants by HPTLC<sup>[24]</sup>

The optimized solvent system selected for the separation of active markers, mangiferin and lupeol present in the

combined extracts of mixture was toluene: ethyl acetate: methanol, 15:3:1.5% v/v/v. The separation was carried out using pre-coated plates containing silica gel 60F<sub>254</sub> on aluminium sheets as the stationary phase. The above mentioned combined plant extracts were examined for mangiferin and lupeol at wavelengths of 270nm and 366nm respectively. The derivatizing agent, Liebermann burchard reagent was used for detecting the spots.

#### Preparation of standard solution of mangiferin and lupeol

10mg each of mangiferin and lupeol (Marker compounds) were transferred into 10ml standard flask and the volume was made up with methanol to 10ml to get a concentration of 1000µg/ml (1:1 ratio concentration). From the above stock solutions, a solution of concentration 200µg/ml was prepared.

#### Preparation of extract mixture

100 mg of *Mangifera indica* and *Hygrophila spinosa* extracts for mixture were transferred separately into 10ml standard flask and the volume was made up with methanol to 10ml to get a concentration of 10000µg/ml (1:1 ratio concentration of mixture).

#### Validation of the method <sup>[26]</sup>

Validation of the HPTLC methods for the selected combined marker mixture was carried out in terms of parameters like linearity, accuracy, limit of detection (LOD), limit of quantification (LOQ), inter and intraday precision, repeatability of sample application and measurement, stability studies and selectivity.

The mixtures of combined markers, mangiferin and lupeol, were prepared in various concentrations and analysed by HPTLC method in order to establish the linear regression data that showed a good linear relationship over a concentration range. From the combined solution of markers prepared, different concentration of mangiferin and lupeol such as 200 – 1000µg/ml and 400 – 800 µg/ml were spotted and chromatograms were recorded. The slope, intercept and correlation co-efficient values were found for each of the calibration graph of the markers.

The limit of detection and limit of quantification of the standards were determined by applying decreasing amounts of the markers in triplicate on the plate. For determining the precision of the method, the analyses of combined markers at different concentrations in the linearity range were carried out for three times on the same day for intraday and three times in a week for interday and their %RSD were calculated.

The repeatability of sample application was assessed by spotting various concentrations of combined marker solutions six times on pre-coated TLC plates and the repeatability of measurement of peak area was assessed after development of the plates and scanning the separated spots for six times and their %RSD were calculated.

Stability of the analyte on the plate was studied at different time intervals and peak areas were compared with the peak area of freshly scanned plate.

#### Recording of the HPTLC chromatogram of the combined extract mixtures

A volume of 10-60µl solutions from the combined mixture of extracts (200 µg/ml) were applied to the plate and chromatograms were developed and then the plates were analysed photo metrically.

Peak areas of the chromatograms of the mixture were compared with those of standard chromatograms and the

amount of markers present in each mixture under study was calculated from the calibration graph.

#### HPLC <sup>[25]</sup>

The optimized solvent system for the mixture was acetonitrile: methanol: 20mM potassium di hydrogen phosphate 30:20:45% v/v/v. The experimental conditions used for the combined plant extract was Phenomenex Luna C<sub>18</sub> column (150×4.6, 5µ) at room temperature, detection of wavelength at 210 nm and a flow rate of 1ml/min. The combined plant extract was analysed by optimized HPLC method. The procedure adopted for the preparation of standard solution of markers and preparation of stock solution of combined extract are given below.

#### Preparation of solution of marker mixture

10mg each of mangiferin and lupeol marker compounds were transferred separately into 10ml standard flask and the volume was made up with methanol to 10ml to get a concentration of 1000µg/ml (1:1 ratio concentration). From the above stock solutions, a mixture having a concentration of 200µg/ml was prepared.

#### Preparation of extract mixture

100 mg of *Mangifera indica* and *Hygrophila spinosa* extracts were transferred into 10ml standard flask and the volume was made up with methanol to 10ml to get a concentration of 10000µg/ml (1:1 ratio concentration of mixture).

#### Validation method of RP-HPLC <sup>[26]</sup>

The validation of the developed method was carried out in terms of parameters like linearity, accuracy, limit of detection (LOD), limit of quantification (LOQ), intra and interday precision, stability studies and selectivity.

The different concentrations of markers were prepared and injected into HPLC system. Linear regression data showed a good linear relationship over a concentration range of mangiferin and lupeol which were 0.6 – 1.4µg/ml, 60-140µg/ml respectively. The peak areas were noted and a linear graph was plotted.

For precision, the analyses of combined markers at different concentrations in the linearity range were done for three times on the same day for intraday and three times in a week for interday and their %RSDs were calculated.

Repeatability of injection was done by injecting six times a combined marker solution of mangiferin and lupeol. Their %RSDs was calculated using peak areas obtained. Specificity was studied by injecting the mobile phase and monitoring for any additional peaks or interference at the retention time (RT) of the marker compounds.

Sample solutions were subjected to stability studies under room conditions. Stability was studied by looking for any change in retention time, resolution, peak shape etc. when compared with the chromatogram of freshly prepared solution. System suitability parameters such as number of theoretical plates (N), resolution (R<sub>s</sub>) and tailing factor were also studied.

#### Recording of HPLC chromatogram of the extracts of the selected plants

A steady baseline was recorded with the fixed chromatographic conditions. This was followed by injecting the solution prepared from the extracts and chromatograms were recorded. The amounts of mangiferin and lupeol present in each of the combined extracts were calculated from the

standard graph which was plotted using peak area of the standard markers against the concentration.

## Results

### Study of biological activity of combination of plant extracts

Evaluation of various combinations of the plant extracts were undertaken to assess their antiarthritic and antigout potentials. It was found that combination of a *Mangifera indica* and

*Hygrophila spinosa* extracts exhibited good activity. The absorbance of the mixture was measured at 359nm for assessing the inhibition of protein denaturation activity, absorbance at 207nm for proteinase inhibitory activity and absorbances at 404nm for evaluation of hyaluronidase inhibitory activity. The percentage inhibitions are presented in Table: 1 to 3. For xanthine oxidase inhibitory assay, absorbance was measured at 290nm and the percentage inhibitions are presented in Table: 4.

**Table 1:** Model 1 - Inhibition of protein denaturation activity

Combination	25µg/ml	50 µg/ml	75 µg/ml	100 µg/ml	IC <sub>50</sub> µg/ml
Mixture	11.9±0.46	28±0.36	36.11±0.14	91±0.09	80±0.08
Diclofenac	42.92±0.03	56.62±0.28	80.24±0.96	94.08±0.04	40±0.35

Mixture *Mangifera indica* and *Hygrophila spinosa*

All the values were expressed as the mean ±SEM (n=6). The data were analysed using one way analysis of variance (ANOVA) followed by Dunnett's test (P<0.05) and were found statistically significant.

**Table 2:** Model 2 - Proteinase inhibitory activity

Combination	10 µg/ml	20 µg/ml	40 µg/ml	80 µg/ml	100 µg/ml	IC <sub>50</sub> µg/ml
Mixture	18.7±0.16	32.1±0.12	59.5±0.22	72±0.28	88.03±0.09	32±0.06
Diclofenac	46.28±0.08	58.58±0.11	77.1±0.20	85.2±0.51	92.68±0.30	13±0.09

Mixture *Mangifera indica* and *Hygrophila spinosa*

All the values were expressed as the mean ±SEM(n=6). The data were analysed using one way analysis of variance (ANOVA) followed by Dunnett's test (P<0.05) and were found statistically significant.

**Table 3:** Model 3 - Hyaluronidase inhibitory assay

Combination	12.5 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml	400 µg/ml	IC <sub>50</sub> µg/ml
Mixture	29.9±0.26	85.95±0.18	84.73±0.21	83±0.36	31.07±0.09	30.7±0.08	16.5±0.20

Mixture *Mangifera indica* and *Hygrophila spinosa*

All the values were expressed as the mean ±SEM(n=6). The data were analysed using one way analysis of variance (ANOVA) followed by Dunnett's test (P<0.05) and were found statistically significant

## Antigout Activity

**Table 4:** Percentage inhibition of combined extracts

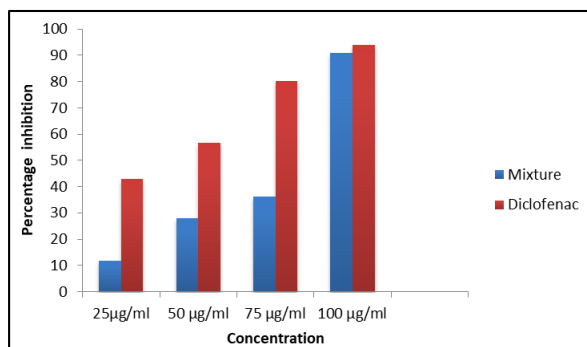
Plant Name	10 µg/ml	20 µg/ml	40 µg/ml	80 µg/ml	100 µg/ml	IC <sub>50</sub> µg/ml
Mixture	26.7±0.42	44.44±0.08	60±0.06	69.5±0.12	78±0.18	28±0.08
Allopurinol	29.39±0.08	32.05±0.09	46.8±0.032	66.8±0.37	91.2±0.052	53±0.28

Mixture *Mangifera indica* and *Hygrophila spinosa*

All the values were expressed as the mean ±SEM (n=6). The data were analysed using one way analysis of variance (ANOVA) followed by Dunnett's test (P<0.05) and were found statistically significant.

### Model 1: Inhibition of protein denaturation

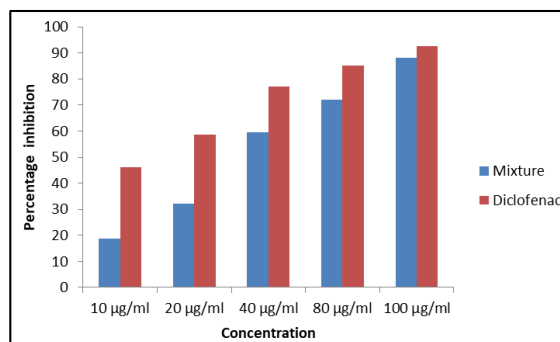
Mixtures of plant extracts were studied for inhibition of protein denaturation activity. When compared to individual plant extracts, combination of extracts was found to offer greater inhibition of protein denaturation. This was evidenced by the remarkable increase in the percentage inhibition. A dose dependent increase in the action was noticed in the study. (Figure 3)



**Fig 3:** Inhibition of protein denaturation activity

### Model 2: Proteinase inhibitory activity

Combination of plant extracts were evaluated for proteinase inhibitory activity. When compared to individual plant extracts, combination of extracts exhibited a higher percentage of inhibitory activity. A dose dependent increase in the activity was observed. Combinations of extracts were found to exhibit synergism in the activity when compared to the individual extracts. (Figure 4).



**Fig 4:** Proteinase inhibitory activity

**Model 3: Hyaluronidase inhibitory assay**

In this assay, combinations of plant extracts were studied for hyaluronidase inhibitory activity. A dose dependent increase in the percentage of inhibition was observed for the mixtures used in the study and the activity was found to be greater than that of the individual plant extracts. As in the case of individual extracts used in the study, the activity of the combined extracts was found to decrease when concentration of the extracts were increased as evidenced by a decrease in percentage of inhibition at 400 µg/ml. Mixture was found to produce a very close IC<sub>50</sub> of 16.5 µg/ml. The concentration of the combined extracts needed to produce IC<sub>50</sub> was much lower compared to those of the individual extracts. From this study it was confirmed that combination of extracts could produce synergism in action (12.5 µg/ml -100 µg/ml) and this may be due to the influence of individual phytoconstituents present in the extract on each other. (Figure 5).

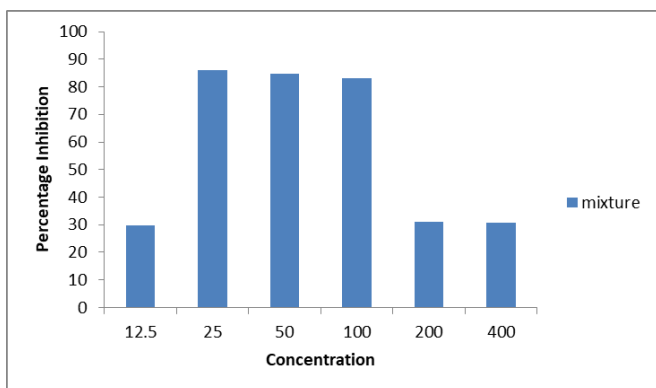


Fig 5: Hyaluronidase inhibitory assay

**Antigout activity****Xanthine oxidase inhibitory assay**

In the xanthine oxidase inhibitory assay, mixture exhibited greater activity as compared to the individual extracts which is evidenced from IC<sub>50</sub> values observed. A dose dependent

increase in the percentage inhibition was noticed for all the concentrations tested. (Figure 6).

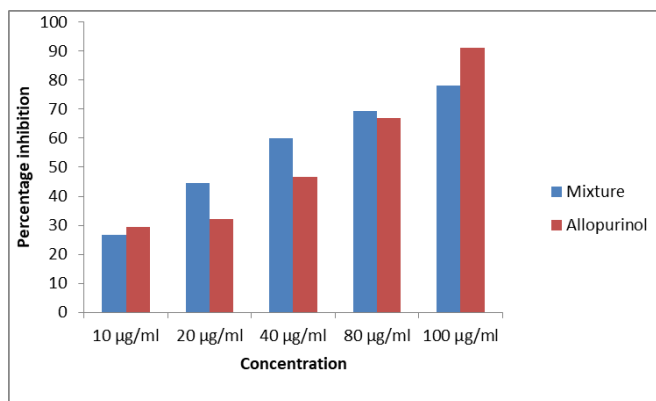


Fig 6: Xanthine oxidase inhibitory assay

**Standardisation of combination of the extracts of selected plants by HPTLC**

The standardisation of combined extract mixtures using relevant markers was carried out using HPTLC. The HPTLC analyses were carried out on the combined plant extracts of *Mangifera indica* and *Hygrophila spinosa* using pre coated plates containing silica gel 60 F<sub>254</sub> on aluminium sheets. The marker mixtures and extract mixtures were found to be soluble in methanol and therefore the stock solutions were prepared using methanol as the solvent. The mobile phase optimized for the mixture was toluene: ethyl acetate: methanol, 15:3:1.5% v/v/v. The mixture was examined at 270nm under UV light after derivatization with Liebermann burchard reagent at 366nm. The chromatograms of marker mixtures and extract mixtures obtained are presented in Figure: 7-9. The amount of the markers present in the combined extract was estimated and the results are presented in Table: 5. The method was validated and the details are presented in Table: 6.

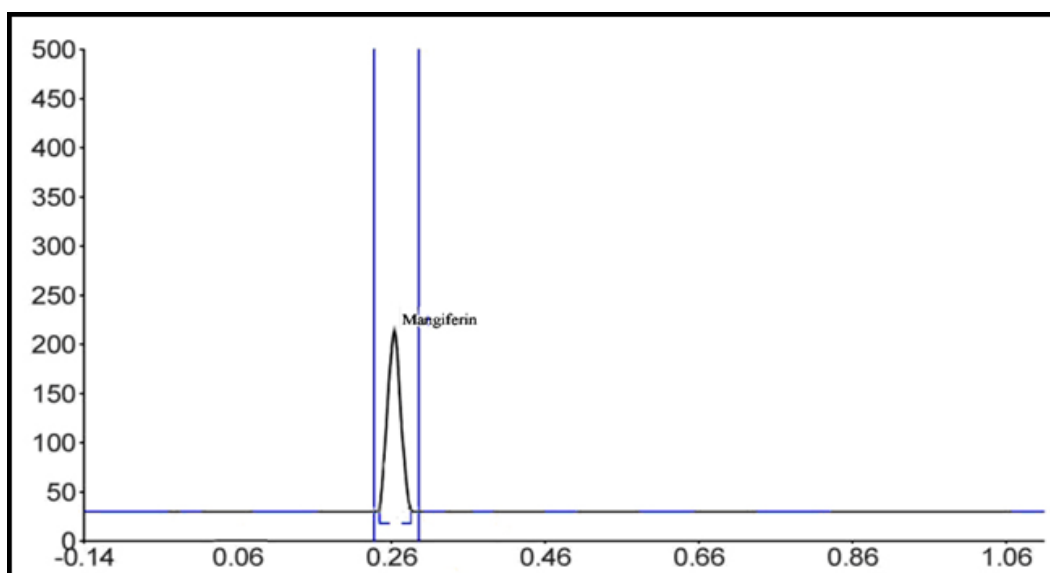


Fig 7: HPTLC chromatogram of mangiferin in mixture

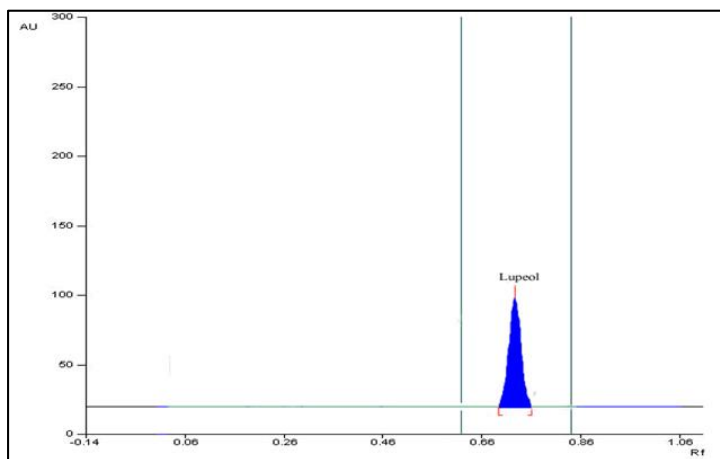


Fig 8: HPTLC chromatogram of lupeol in mixture

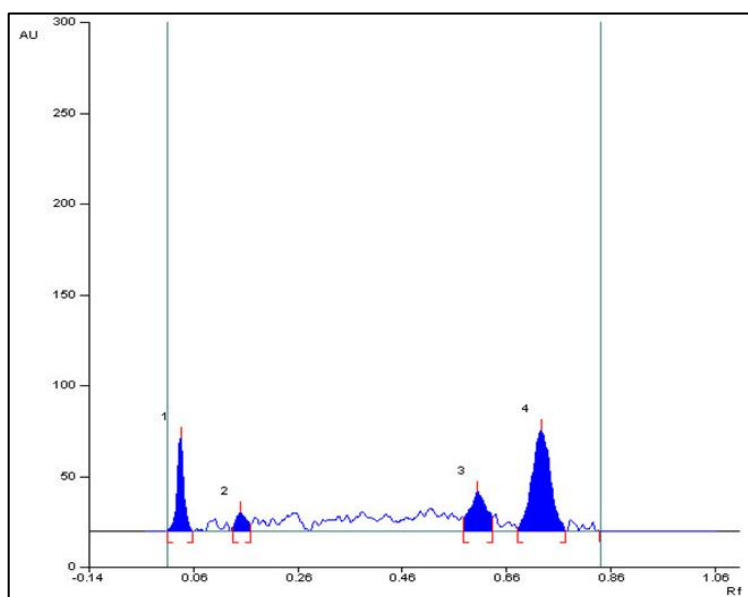
Fig 9: HPTLC chromatogram of the extract mixture containing *Mangifera indica* and *Hygrophila spinosa*

Table 5: Amount of markers present in the extract mixture

Amount estimated / 10 mg	HPTLC		HPLC	
	Mangiferin (mg)	Lupeol (mg)	Mangiferin (mg)	Lupeol (mg)
	0.330	2.474	0.312	2.142

Table 6: Validation parameters of HPTLC and HPLC method for the estimation of individual markers

Parameters	HPTLC		HPLC	
	Mangiferin	Lupeol	Mangiferin	Lupeol
Linearity	200-1200	400-800	0.2-1.4 µg/ml	60-140
Retention time(RT)	(ng/spot)	(ng/spot)	4.002	µg/ml
Slope	-	-	42574.5	163.83
Intercept	6.834	1.784	- 2155.7	121.1
R	-251.735	-114.550	0.9938	0.9929
LOD (ng/spot)	0.9998	0.9912	0.05 µg/ml	5µg/ml
LOQ (ng/spot) Precision (%RSD)	60ng/spot	70ng/spot	0.2 µg/ml	20µg/ml
Intraday	200ng/spot	100ng/spot		
Interday	1.47	1.21	0.49	0.79
Repeatability	1.5	1.5	0.57	1.20
of application	1.58	1.81	1.06	0.65
Repeatability	1.79	1.09	-	-
of measurement				
Stability on plate (min)	120	30	5	300

### Standardization of combined extracts of the selected plants by HPLC

The HPLC analyses were carried out on the selected

combined extracts of *Mangifera indica* and *Hygrophila spinosa* on Phenomenex Luna C<sub>18</sub> column. The standard stock solutions of marker mixtures and extract mixtures were

prepared using methanol in which they were soluble. The optimized solvent system for the mixture was found to be acetonitrile: methanol: 20mM potassium dihydrogen phosphate 30:20:45%v/v/v. The flow rate was fixed as 1ml/min. The detection wavelength for mixture used was 210nm. The chromatograms for the marker mixtures and extract mixture are presented in Fig: 10 and 11. The estimated values for the amount of markers present in the mixtures are presented in Table: 5. The method was validated and the details are presented in Table: 6.

### Conclusion

Investigation of the biological activities and standardisation of combination of the extracts of the two selected medicinal plants were carried out in the present work. The study was conducted on the extracts of the bark and aerial parts of the plants *Mangifera indica* and *Hygrophila spinosa* respectively. A very high percentage (nearly 60 to 90%) of antiarthritic activity and antigout activity was found when inhibition of protein denaturation, proteinase inhibition and hyaluronidase inhibitory activity and xanthine oxidase activity were evaluated by *in vitro* method for the combined plant extracts. These combined plant extract mixtures were standardised and quantified by newly developed HPTLC and HPLC methods. The newly developed HPTLC and HPLC techniques for the estimation of marker constituents present in the combined extracts that showed good antiarthritic and antigout activities can be utilized in the routine analysis and standardization of mixture of medicinal plant extracts.

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